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Article

RNAi-based gene therapy targeting ZGPAT promotes EGF-dependent wound healing

Jiwon Lee[1], Woo Rin Lee[2]
[1]Chadwick International, 45, Art center-daero 97 beon-gil, Yeonsu-gu, Incheon, Republic of Korea
[2]Department of Biological Science, University of Suwon, Wau-ri, Bongdam-eup, Hwaseong, Gyeonggi-do, Republic of Korea

SUMMARY

Wound-healing is a multi-dynamic skin repair process that involves a sequence of events, such as inflammation, proliferation, and migration of different cell types like fibroblasts. Epidermal growth factor (EGF) is an excellent wound healing agent, and epidermal growth factor receptor (EGFR) becomes particularly important for wound healing. Zinc Finger CCCH-type with G-Patch Domain Containing Protein (*ZGPAT*), encodes a protein that has its main role as a transcription repressor by binding to a specific DNA sequence and is capable of inhibiting the expression of EGFR. We hypothesize that targeting *ZGPAT* with RNA-interference-based gene therapy will improve wound healing by alleviating inhibition of EGFR expression. We used EGF and siRNA to inhibit *ZGPAT* in a normal skin cell line, Detroit 551 with migration assay as a model for wound healing. The aim of the study was to find out whether inhibiting *ZGPAT* will expedite the wound healing process by accelerating cell migration. We found that downregulation of *ZGPAT* by siRNA increased EGFR expression in Detroit 551. Further, simultaneous treatment of siZGPAT and EGF significantly increased cell migration in Detroit 551 cells. This novel combination of treatment can provide a key to the development of wound healing strategies in medicine and cellular biology.

INTRODUCTION

Wound by definition is damage to the integrity of biological tissue including skin, mucous membranes, and organ tissues following various traumas such as surgery, a cut, chemicals, heat/cold, friction, and pressure or as a result of diseases such as leg ulcers or cancers (1). Wound healing is a multi-dynamic biological skin process consisting of precisely and highly regulated overlapping phases: inflammation, proliferation, and migration of different cell types as fibroblasts (2). Delayed skin wound healing can cause infections, organ damages, and so on. Moreover, it can require surgery and extended hospitalization especially in aged and diabetic patients (3).

Wound healing assay is a technique used to analyze cell migration. In collective cell migration, the cells move as a group, and are either tightly or loosely held together. This collective cell migration constructs almost all of the living tissues (4). The cell-cell junction enables the cohesion and signaling between cells and produces cell polarity. Cadherin and transmembrane proteins form a junction between cells. The gradients of extracellular signaling molecules can control cell movement. When some or all the cells sense these, cell to cell interactions are triggered. Research on cell migration as epithelial sheets has found that during embryonic morphogenesis, only the leader cells detect and move along the gradient (4). The adjoining cells follow the leader cells by the cell-to-cell interactions (5). Understanding the molecular mechanism behind the cell migration and wound-healing process could translate to faster healing and avoiding infection or scarring in the clinic.

Epidermal growth factor (EGF) is as an excellent wound healing agent due to its ability to stimulate skin cell growth, proliferation, and differentiation (6). However, transdermal delivery of EGF faces significant challenges due to the drug's short half-life and lack of efficient formulation that can carry the drug through layers of keratinocytes (7). Thus, some studies have investigated manipulating the expression of its receptor, epidermal growth factor receptor (EGFR).

The EGFR, EGF receptor, is important for wound healing. EGF and EGFR activate epidermal and dermal regeneration (8). Skin is composed of three layers: epidermis, dermis, and subcutaneous tissue. Epidermis is the outermost layer and subcutaneous tissue is the lowermost one (4). Thus, wounds can be treated by targeting EGF or EGFR (9). The signaling pathways activated by EGFR are categorized into groups that include cell migration, proliferation, cytoprotection, and epithelial-mesenchymal transition (EMT). The migration and proliferation pathways must be activated to heal the wound damage (10). The expression of EGFR influences the responsiveness of cells to the EGF signals. Transcriptional regulators can alter EGFR expression.

Zinc Finger CCCH-type with G-Patch Domain Containing Protein (*ZGPAT*) encodes a protein that functions as a transcription repressor by binding to a specific DNA sequence (5'-GGAG[GA]A[GA]A-3') (11). It can inhibit the expression of EGFR. Also, ZGPAT inhibits cell proliferation and suppresses breast carcinogenesis (12). In a tumor, cell proliferation appears disorganized, but wounding triggers a coordinated and synchronized proliferative signals. Therefore, the sustained proliferative signaling and the activation of cell

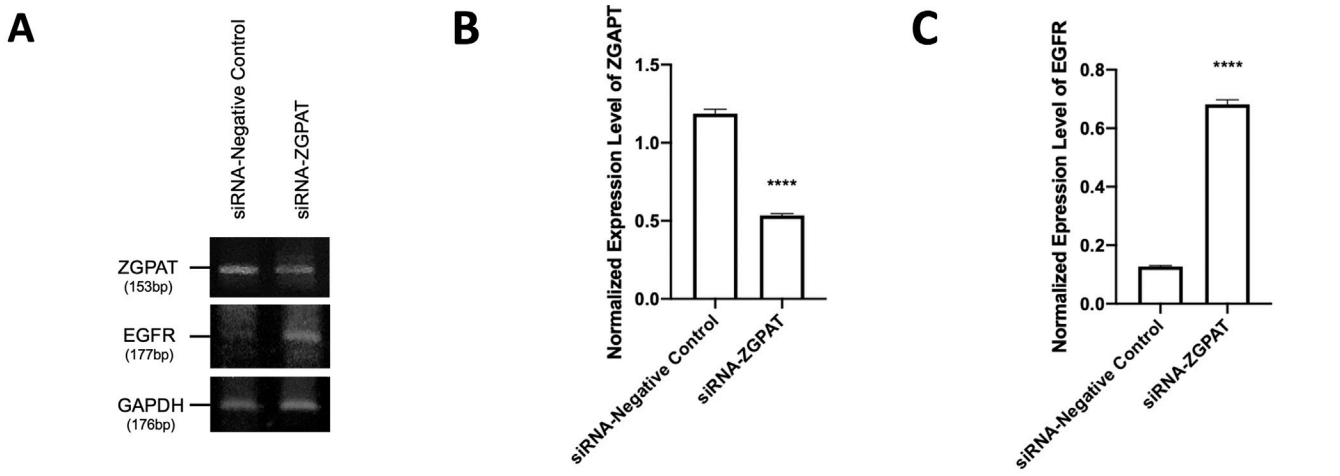

Figure 1. ZGPAT targeting siRNA decreases ZGPAT mRNA expression level and increases EGFR expression. (A) Agarose gel electrophoresis of RT-PCR products of siCon (siRNA-Negative Control) and siZGPAT (siRNA-ZGPAT) samples targeting ZGPAT, EGFR, and GAPDH cDNA (ZGPAT annealing temperature = 50°C, EGFR annealing temperature = 57.5°C, GAPDH annealing temperature = 55°C). **(B)** Quantification of normalized ZGPAT expression level by GAPDH expression level based on agarose gel electrophoresis of RT-PCR products. **(C)** Quantification of EGFR expression level by GAPDH expression level based on agarose gel electrophoresis of RT-PCR products. The mean and standard deviation was represented in the graph. $n = 3$, unpaired t-test $p < 0.0001$ (****).

migration may induce faster wound healing.

One method of influencing the effectiveness of ZGPAT is RNA interference. RNA interference (RNAi) is a naturally occurring process of fighting off invasive external genes. Small interfering RNA (siRNA) can silence the expression of target genes. This RNA uses sequence-specific targeting to mediate targeted messenger RNA (mRNA) degradation. siRNA allows almost any gene to be targeted because it works through Watson-Crick base pairing with mRNA (13). siRNA operates by selecting the correct nucleotide sequence along the targeting mRNA. Recently, RNAi is widely accepted as an effective pharmaceutical practice. The United States Food and Drug Administration (FDA) has approved RNAi-based therapeutics for the treatment of genetic disease such as hereditary amyloidogenic transthyretin (hATTR) amyloidosis and acute hepatic porphyria (AHP) (14, 15). Therefore, RNAi presents a promising mechanism for altering transcriptional regulators like ZGPAT. We hypothesized that if EGFR expression is upregulated by inhibiting *ZGPAT* gene expression through RNAi, then the rate of cell migration would increase. To test this hypothesis, we targeted the *ZGPAT* gene via RNAi, and analyzed its subsequent effect on EGFR expression and the cell migration and proliferation of a normal skin cell line, Detroit 551. Here, we show that siRNA-mediated knockdown of *ZGPAT* increased the cell migration capacity of Detroit 551 cells *in vitro*. Our results also suggested that *ZGPAT* affects EGFR signaling, providing a rationale for targeting *ZGPAT* by RNAi for the promotion of wound healing.

RESULTS

The purpose of this experiment was to investigate whether targeting the *ZGPAT* gene with siRNA will increase the expression of EGFR. To test this, we used polymerase chain reaction (PCR) in the Detroit 511 cell line treated with siRNA-*ZGPAT in vitro*. In the siRNA-*ZGPAT* sample, the *ZGPAT* band intensity is suppressed, while the EGFR band intensity is increased compared to the siRNA-Negative control sample (**Figure 1A**). The densitometry analysis indicated that cells treated with siRNA-*ZGPAT* had an approximately 50% reduction in *ZGPAT* gene expression, and an approximately 6-fold increase in EGFR gene expression (**Figure 1B**, **C**). The band intensity of ZGPAT and GAPDH is quantified (**Table 1**). Overall, the result suggests that the downregulation of *ZGPAT* by siRNA increased the expression of EGFR.

We hypothesized that EGF-dependent cell migration will increase after *ZGPAT* gene expression was inhibited. Therefore, we measured the rate of cell migration for each sample which had four different treatment conditions: no treatment (control), EGF only, EGF + siNegative control, and EGF + siZGPAT. All four images taken at 0 hour showed large gaps in the middle of the image (**Figure 2**). The blank spaces represent the wound created by scratching the cell in a straight line with a pipette tip. All four images taken after 24 hours depicted cells' movement toward the blank space to

Table 1. Band intensity measurements from densitometry.

	Sample #	GAPDH	EGFR	ZGPAT	ZGPAT/GAPDH	EGFR/GAPDH
siRNA-Negative control	1	5200.246	681.012	6129.912	1.179	0.131
	2	5120.231	632.013	6231.594	1.217	0.123
	3	5410.634	691.156	6312.125	1.178	0.128
siRNA-ZGPAT	1	6289.146	4242.903	3327.941	0.529	0.675
	2	6164.134	4312.012	3241.553	0.524	0.700
	3	6321.531	4245.535	3463.521	0.548	0.672

close the wound (**Figure 2**). When *ZGPAT* was suppressed in the presence of EGF, the migration rate increased two-fold (**Figure 3**). Overall, when siRNA targeting *ZGPAT* and EGF are co-treated, cell migration was increased. Increased EGFR expression may increase the sensitivity to EGF, which activates EGF-EGFR signaling. Therefore, the combined treatment of siZGPAT and EGF may enhance wound healing by increasing cell migration.

DISCUSSION

Wound-healing is a dynamic skin repair process that involves a sequence of events, such as inflammation, proliferation, and migration of different cell types like fibroblasts (2). Delayed skin wound healing can cause infections, organ damages, and so on. Moreover, it can require surgery and extended hospitalization especially in aged and diabetic patients (3). The aim of our study was to find out whether inhibiting *ZGPAT* will expedite the wound healing process by accelerating cell migration.

The *ZGPAT* gene is a transcriptional repressor of EGFR. The production of EGFR is restricted in the presence of high levels of *ZGPAT* (14). Therefore, it is possible that when the siRNA of *ZGPAT* was delivered into the cells, the *ZGPAT* protein may also be decreased, so that EGFR gene expression level increased. Therefore, it is possible that a greater amount of EGF from the extracellular matrix was able to bind to the EGFR (16). Upregulation of EGFR may activate the signaling cascade that induces rapid cell migration (**Figure 4**). Overall, since EGF is commonly found in wound ointments, the simultaneous treatment of siRNA targeting *ZGPAT* (siZGPAT) and EGF may enhance wound healing by increasing cell migration.

When siRNA targeting *ZGPAT* and EGF were co-treated, cell migration was increased *in vitro*. This increase is possibly due to the increased binding of EGF to EGFR by inducing high EGFR expression through *ZGPAT* knockdown. It is also possible that *ZGPAT* may increase the sensitivity of EGF, which activates EGF-EGFR signaling. EGF is mainly contained in common wound ointments (17). A low dose of EGF was used in our cell migration assay even though EGF alone also enhanced cell migration (**Figure 3**). Therefore, any additional effects on cell migration by *ZGPAT* knockdown would not be masked by the dramatic effects of high-dose EGF alone. However, siZGPAT alone control was not investigated in this research. Indeed, we observed a marked increase in cell migration with siZGPAT plus EGF over EGF alone (**Figure 3**).

The clinical application of siRNA is hindered by poor stability and the possibility of unexpected and unwanted effects as it may affect the off-targets (18). Theoretically, siRNA properly executes its function only when the base pairs of the antisense strand are completely paired with the base pairs of the target mRNA. Previous research indicates that a few mismatches produced by RNA-induced silencing complex (RISC) can cause unwanted inhibition of the expression of the

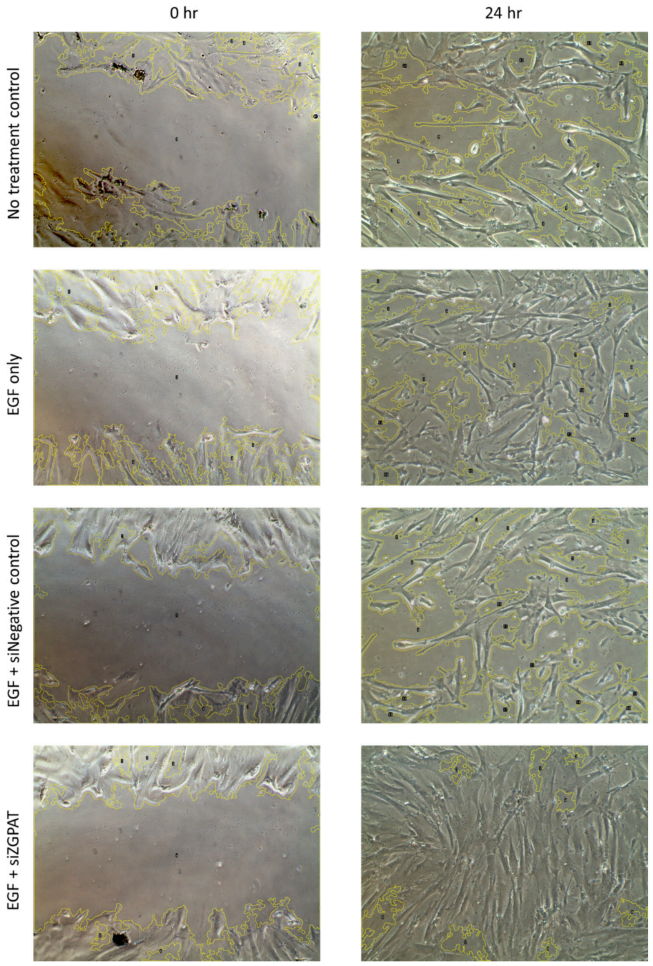

Figure 2. *ZGPAT* inhibition increases EGF-dependent cell migration in Detroit 551. Wound healing assay was performed to evaluate the migration of cells.

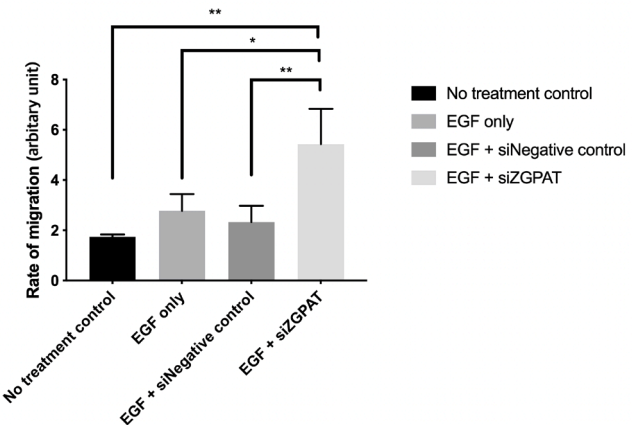

Figure 3. Quantification of EGF-dependent cell migration after ZGPAT gene expression was inhibited with EGF treatment. Rate of migration = (Area without cells at 0 hr after transfection) / (Area without cells at 24 hr after transfection), one-way ANOVA with Tukey's multiple comparison correction, $n = 3$, p-value < 0.05 (*), p-value < 0.01 (**).

target genes or the inhibition of the expression of unrelated genes (16). In addition, unmodified siRNA can activate Toll-like receptor 3 (TLR3), which can cause negative impacts on the blood and lymphatic systems (19). These are the limitations for the RNA-based gene therapies that are still in infancy and face multiple challenges in safe and efficacious clinical applications.

The reliability and accuracy of this experiment's results could be increased through the following future research. First, the off-target effect of siRNA can be eliminated by testing more siRNAs that target different positions of *ZGPAT* mRNA to confirm that the increased cell migration is not a result of the off-target effect. Secondly, since the previous experiment was conducted on normal human skin cells, the same experiment could be done in the mouse model system to confirm the effect of *ZGPAT* inhibition on the rate of wound healing. Lastly, to investigate whether *ZGPAT* can bind to regulatory regions near the EGFR gene to control EGFR transcriptional output, a chromatin immunoprecipitation sequencing (ChIP-seq) experiment using anti-*ZGPAT* antibody, and RNA sequencing (RNA-seq) experiment comparing EGFR mRNA expression in both control and siZGPAT transfected conditions should be conducted in the future. In conclusion, this combination of treatment increases cell migration and enhanced the wound healing. This method can provide a key to the development of wound healing strategy.

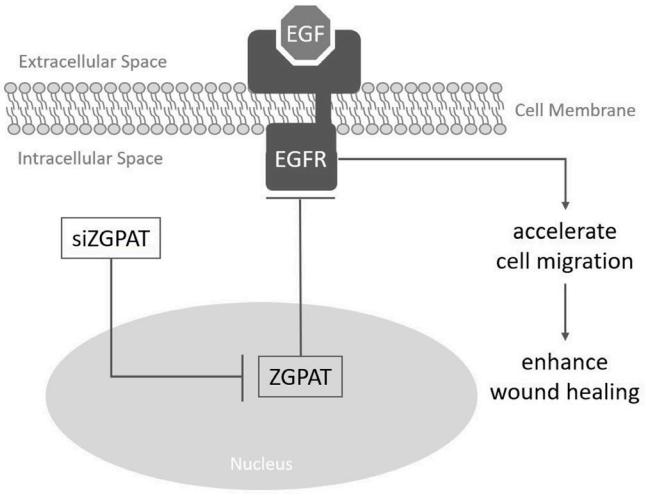

Figure 4. Proposed molecular mechanism that after siZGPAT is transfected in the cells, it enhances wound healing by activation of EGF-EGFR signaling. *ZGPAT* repress the transcription of EGFR promoter. siZGPAT inhibits *ZGPAT* expression resulting increased expression of EGFR. This leads to accelerate cell migration and enhanced wound healing.

METHODS

Cell culture

Detroit 551 cell line was purchased from Korea Cell Line Bank. Detroit 551 cells were maintained in RPMI 1640 media (Gibco) in a 37 ˚C CO_2 incubator (Eppendorf). Before the experiment, cell media was removed by suction, and 400 µL of 0.25% trypsin-EDTA (Gibco) was added to the cells to detach the cells from the culture plate. Cells were incubated for 5 to 10 min at 37 ˚C.

siRNA transfection

The Detroit 551 cells were seeded in a 24-well culture plate before the transfection. Of the 500 µL of resuspended media with cells, 50 µL was moved to the siCon (the same nucleotides as the siZGPAT but scrambled) in the 24-well culture plate, and another 50 µL was moved to the siZGPAT (siRNA that targets *ZGPAT* gene). 350 µL of media was added to each well to create a total of 400 µL media with cells. Then, the mixture of RNAimax transfection reagent (Invitrogen) and siRNA was prepared. Two 1.5 mL tubes were prepared with 50 µL of media. 1.3 µL of Lipofectamine RNAimax reagent was added to one tube, while 0.3 µL of siRNA *ZGPAT* was added to the other tube. The two solutions were mixed to form a solution close to 100 µL. The mixed solution was incubated for 5 min for the reagent and siRNA to produce a complex. This solution was added to 400 µL in siCon to produce a total of 500 µL media. Using the same process, another 100 µL mixed solution of siRNA and reagent were prepared and added to 400 µL in siZGPAT to produce a total of 500 µL media. All cells were then incubated for 48 hr. All siRNAs used in this study were synthesized by Bioneer.

Total RNA extraction

Total RNA from each sample was purified using the RNA-spin Total RNA Extraction Kit (Intron). Detroit 551 cells were harvested, and the media was removed by centrifuging at 13,000 g for 10 sec. We added freshly prepared R-buffer to the tube and vortexed at room temperature for 30 seconds. The lysate was incubated at room temperature for 5 to 10 min and then vortexed. We mixed the cell lysates with 70% ethanol and, loaded them on the column, and centrifuged at 13,000 g for 30 sec. The flow-through was discarded. We washed the columns with Washing Buffer A at 13,000 g for 30 sec, then Washing Buffer B. The flow-through was discarded after each washing step, and the spin column was placed back in the same 2 mL collection tube. The tube was centrifuged at 13,000 g for 2 min to dry the RNA-spin membrane. The column was placed in a clean 1.5 mL microcentrifuge tube. We added Elution buffer directly onto the cell membrane, incubated it at room temperature for 1 min, and centrifuged the sample at 13,000 g for 1 min to elute.

cDNA synthesis

Two PCR tubes were prepared. 14 µL of siCon RNA buffer, 2 µL of RT buffer, 1 µL of reverse transcriptase (Enzynomics), 2 µL of dNTP, and 1 µL of primer were added into the siCon PCR tube. 14 µL of siZGPAT RNA, 2 µL of RT buffer, 1 µL of reverse transcriptase, 2 µL of dNTP, and 1 µL of primer were added into the siZGPAT PCR tube. Using a 10 µL pipette, each solution was mixed by pipetting. Both PCR tubes were

centrifuged for 5 to 10 sec for the liquid to accumulate in the bottom of the tubes. Then, both PCR tubes were placed in the PCR machine at 25 ˚C for 10 min, 42 ˚C for 60 min, 95 ˚C for 5 min, and 4 infinitely.

Polymerase chain reaction (PCR)

17 µL of water, 1 µL of forward primer, 1 µL of reverse primer, and 1 µL of cDNA were prepared. Four different PCR tubes were prepared. 750 µL of water was added to each forward and reverse primer to make 20 pmol/µL concentration. Both the forward and reverse primers were placed in the vortexer for 10 sec to mix the solutions. 17 µL of water was added to each forward and reverse PCR tube. 1 µL of forward primer was added to the forward PCR tubes, and 1 µL of reverse primer was added to the reverse PCR tubes. 1 µL of cDNA was added to each of the four PCR tubes. All four PCR tubes were placed in the vortexer for 10 sec, centrifuged for another 10 sec, and placed in the PCR machine to start the PCR reaction. The PCR reaction was performed as the following: 1) 95 ˚C for 3 min, 2) 95 ˚C for 30 sec, 3) 55 ˚C for 30 sec, 4) 72 ˚C for 1 min, 5) repeat step 2 to 4 for 34 times, 6) 72 ˚C for 5 min, 7) 12 ˚C infinitely. To optimize the PCR condition, the annealing temperatures were set differently for each sample: 50 ˚C for *ZGPAT*, 57.5 ˚C for EGFR, and 55 ˚C for GAPDH. The following primer sequences were used to amplify target genes: *ZGPAT*_F: 5' - CCTGGGGCACTCTGGAGTAT - 3', *ZGPAT*_R: 5' - CCTTAAAGCGGCACTTTCCCT - 3', EGFR_F: 5' - AGGCACGAGTAACAAGCTCAC- 3', EGFR_R: 5' - ATGAGGACATAACCAGCCACC - 3' GAPDH_F: 5' - TGGAGAAGGCTGGGGCTCAT - 3', GAPDH_R: 5' - GACCTTGGCCAGGGGTGCTA - 3'. All PCR reaction buffer and enzymes and primers were purchased from Bioneer.

Agarose electrophoresis gel analysis

1.2 g of agarose powder (Intron) was mixed with 100 mL Tris-Borate-EDTA (TBE) buffer to make 1.2 % agarose gel. The mixed solution was heated up in a microwave until all agarose powder was dissolved completely. 5 µL of nucleic acid staining solution (Intronbio) was added into the solution for DNA staining. Then, the agarose solution was poured into the gel caster (Mupid). When the gel was completely solidified, the agarose gel was placed in the agarose gel tank (Mupid). All samples were loaded inside the agarose well. One hundred volts was applied to the gel tank for 25 min. After running the gel electrophoresis, agarose gel was analyzed under a blue light illuminator (B-box).

Cell migration

Detroit 551 cells were seeded on a 6-well culture dish (SPL). The cells were incubated for 6 hr in a CO2 incubator allowing cells to adhere. The cells were treated with siZGPAT or siControl for 48 hours, before the addition of EGF (100 ng/mL) and subsequent 24-hour cell migration assay. Then, the cells were scraped with the pipette tip in a straight line to create a wound. The detached cells were washed with PBS. The RPMI1640 media supplemented with 1% FBS (Sigma) and EGF (100 ng/mL) (Invitrogen) was added. The images of the migrated cells were acquired by Eclipse upright microscope (Nikon).

Rate of cell migration quantification

After the image was loaded on the ImageJ program (1.48v), cell-free area was analyzed by the Montpellier Resources Imagerie (MRI) wound healing tool. The following setting was used in the imageJ: Find edges method, variance filter radius 1, threshold 5, min size 1000. The rate of migration was calculated as the following equation: Area without cells at 0 hr after transfection / Area without cells at 24 hr after transfection.

Band intensity quantification by densitometry analysis

ImageJ program was used to quantify the band intensity by densitometry. GAPDH intensity was used for normalizing the target gene expression level. "Analyze" and "Gel" functions were used to select the area by drawing a rectangle. After all areas were selected, the area of the band intensity was measured by "Plot Lane" function. The area of background intensity was subtracted by drawing the line from the plot. "Wand (tracing tool)" was used to measure the final area. The expression level of *ZGPAT* and EGFR was normalized by the expression level of GAPDH. The band intensity of *ZGPAT* and EGFR was divided by GAPDH for normalization.

Statistical analysis

All statistical analysis (unpaired *t*-test, one-way ANOVA with Tukey's multiple comparison correction) was performed using Prism 8.

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